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MRP8, ATP-binding Cassette C11 (ABCC11), Is a Cyclic Nucleotide Efflux Pump and a Resistance Factor for Fluoropyrimidines 2′,3′-Dideoxycytidine and 9′-(2′-Phosphonylmethoxyethyl)adenine*

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MRP8 (ABCC11) is a recently identified cDNA that has been assigned to the multidrug resistance-associated protein (MRP) family of ATP-binding cassette transporters, but its functional characteristics have not been determined. Here we examine the functional properties of the protein using transfected LLC-PK1 cells. It is shown that ectopic expression of MRP8 reduces basal intracellular levels of cAMP and cGMP and enhances cellular extrusion of cyclic nucleotides in the presence or absence of stimulation with forskolin or SIN-1A. Analysis of the sensitivity of MRP8-overexpressing cells revealed that they are resistant to a range of clinically relevant nucleotide analogs, including the anticancer fluoropyrimidines 5′-fluorouracil (~3-fold), 5′-fluoro-2′-deoxyuridine (~5-fold), and 5′-fluoro-5′-deoxyuridine (~3-fold), the anti-human immunodeficiency virus agent 2′,3′-dideoxycytidine (~6-fold) and the anti-hepatitis B agent 9′-(2′-phosphonylmethoxynyl)adenine (PMEA) (~5-fold). By contrast, increased resistance was not observed for several natural product chemotherapeutic agents. In accord with the notion that MRP8 functions as a drug efflux pump for nucleotide analogs, MRP8-transfected cells exhibited reduced accumulation and increased efflux of radiolabeled PMEA. In addition, it is shown by the use of *in vitro* transport assays that MRP8 is able to confer resistance to fluoropyrimidines by mediating the MgATP-dependent transport of 5′-fluoro-2′-deoxyuridine monophosphate, the cytotoxic intracellular metabolite of this class of agents, but not of 5′-fluorouracil or 5′-fluoro-2′-deoxyuridine. We conclude that MRP8 is an amphipathic anion transporter that is able to efflux cAMP and cGMP and to function as a resistance factor for commonly employed purine and pyrimidine nucleotide analogs.

Cellular extrusion of cyclic nucleotides has been described in prokaryotic and eukaryotic cells (1–4). This process provides extracellular cAMP involved in intercellular signaling, as determined for *Dictyostelium discoideum*, in which cAMP effluxed by solitary amoebae under low nutrient conditions mediates cellular aggregation and differentiation, and has also been proposed as a potential mechanism that may contribute to the attenuation of intracellular signaling mediated by these second messengers (5). Investigations employing cultured cells and membrane vesicle preparations have established that cyclic nucleotide efflux is energy-dependent, and the susceptibility of this process to inhibition by antagonists of organic anion pumps indicates that it is mediated by amphipathic anion transporters (2, 3, 6–16). Recently, insights into the identities of the cellular components that mediate cyclic nucleotide efflux have come from studies of the MRP[1] family of ABC transporters. MRP4 and MRP5, two members of this extended family of amphipathic anion transporters (17), have been determined to be competent in the transport of cyclic nucleotides (18–20). By contrast, other characterized MRP family members are able to transport a variety of lipophilic anions, such as glutathione and glucuronic acid conjugates, but not cyclic nucleotides (17). In addition to its ability to efflux cyclic nucleotides, MRP4 is also able to mediate the transport of methotrexate, reduced folates, estradiol 17-β-D-glucuronide and DHEAS (19, 21–23), and both MRP4 and MRP5 have the facility for conferring resistance to certain purine nucleotide analogs, such as PMEA, an amphipathic antiviral agent used in the treatment of hepatitis B infections, and 6-mercaptopurine, an anticancer nucleobase analog (21, 24, 25).

MRP8, a newly identified cDNA, was recently assigned to the MRP family based upon analyses of its predicted protein (26–28). MRP8 resembles MRP4 and MRP5 in that it lacks a third (N-terminal) membrane-spanning domain that is present in MRP1, MRP2, MRP3, MRP6, and MRP7. In addition, sequence comparisons with MRP family members indicate that it most closely resembles MRP5 (26, 27). These features suggest the possibility that MRP8 might represent a component of the efflux system for cyclic nucleotides. However, the functional properties of MRP8 have not been determined with respect to either its substrate selectivity or drug resistance capabilities. Here we examine the functional characteristics of MRP8 in transfected LLC-PK1 cells. It is demonstrated that MRP8 is an efflux pump for cAMP and cGMP and that it not only is capable of conferring resistance to the purine nucleotide analog PMEA but also has the ability to function as a resistance factor for fluoropyrimidines, a widely employed class of antineoplastic agents, and the anti-AIDS drug 2′,3′-dideoxycytidine.

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[1] The abbreviations used are: MRP, multidrug resistance-associated protein; ABC, ATP-binding cassette; PMEA, 9-(2-phosphonylmethoxynyl)adenine; bis-POM-PMEA, bis(pivaloyloxymethyl)-PMEA; ddC, 2′,3′-dideoxycytidine; 5-dFUrd, 5′-deoxy-5′-fluorouridine; 5-FdUMP, 5′-fluoro-2′-deoxyuridine monophosphate; 5-FUra, 5′-fluorouracil; 5-FdUrd, 5′-fluoro-2′-deoxyuridine.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]Bis-POM-PMEA (2 Ci/mmol), [^{3}H]5′-fluorouracil (5-FUra) (17.5 Ci/mmol), [^{3}H]5′-fluoro-2′-deoxyuridine (5-FudR) (13.9 Ci/mmol), and [^{3}H]5′-fluoro-2′-deoxyuridine 5′-monophosphate ([^{3}H]5-FdUMP) (10.7 Ci/mmol) were purchased from Moravek (Brea, CA). Unlabeled 2′,3′-dideoxycytidine (ddC), 3′-azido-3′-deoxythymidine, 5-FUra, 5-FdUrd, 5′-fluoro-5′-deoxyuridine (5-dFUrd), 6-thioguanine, 2′-chloro-2′-deoxyadenosine, doxorubicin, paclitaxel, and vincristine were obtained from Sigma. Etoposide was obtained from Bristol-Myers Squibb Co. PMEA was obtained from Gilead (Forest City, CA). 2′,3′-Dideoxy-3′-thial-cytidine was provided by the National Institutes of Health AIDS program. Forskolin and SIN-1A were obtained from the Sigma and Cayman Chemical (Ann Arbor, MI), respectively.

Expression Vector Construction, Transfection, and Cell Culture—Two overlapping cDNA fragments that encode the MRP8 open reading frame were prepared by PCR using rapid amplification of cDNA ends (RACE) ready prostate library (Stratagene, La Jolla, CA) and oligonucleotide primers derived from the reported sequence (26) and were assembled into Bluescript SK(−) (Stratagene). The predicted coding sequence was identical to that reported by Berra *et al.* (26). The MRP8 cDNA was inserted into pcDNA3.1 (Invitrogen) to create pcDNA-MRP8. LLC-PK1 cells grown in M199 medium supplemented with 10% fetal bovine serum were electroporated with 10 μg of pcDNA-MRP8 or parental plasmid, and G418-resistant colonies were isolated. For expression in insect cells the MRP8 coding sequence was inserted into PVL1392, and production of baculovirus and infection of insect cells were accomplished according to the manufacturer's directions (Pharmingen).

Generation of MRP8 Polyclonal Antibody and Immunoblot Analysis—A cDNA fragment encoding amino acids 746–804 of MRP8 was inserted downstream of the glutathione *S*-transferase coding sequence in PGEX2T, and the fusion protein was purified by the use of glutathione-Sepharose beads (Amersham Biosciences). Rabbits were immunized with the fusion protein, and the specificity of the resulting antisera was confirmed by immunoblot analysis of cellular lysates prepared from insect cells infected with MRP8 baculovirus. Proteins were separated by 8% SDS-PAGE and electrotransferred to nitrocellulose filters using a wet transfer system as described previously (29, 30). MRP8 was detected using polyclonal MRP8 antibody (1:500) and horseradish peroxidase-conjugated antibody (Amersham Biosciences).

Measurement of Cyclic AMP and Cyclic GMP—cAMP was measured with the cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer's recommendations. Cells were plated in triplicate at a density of 8000 cells/well in 96-well plates. The following day cells were lysed, and an aliquot was transferred to a second plate for the measurement of cAMP. For stimulation experiments, cells were treated with 50 μM forskolin, and cAMP measurements were performed at various time points over a 60-min time period. Extracellular cAMP was determined by subtracting intracellular cAMP from total (combined intracellular + extracellular) cAMP. cGMP levels were similarly measured using the cGMP enzyme immunoassay system (EIA, Amersham Biosciences), except that stimulation was accomplished using 50 μM SIN-1A.

Analysis of Drug Sensitivity—Drug sensitivity was analyzed by the use of a tetrazolium salt microtiter plate assay (CellTiter 96 Cell Proliferation Assay, Promega, Madison, WI). Cells were seeded at a density of 5000 cells/well in 96-well dishes in complete medium, and drugs were added at various concentrations on the following day. Growth assays were performed after 72 h of growth in the presence of drugs.

Accumulation and Efflux of [^{3}H]bis-POM-PMEA—For accumulation experiments, cells were plated in triplicate at a density of 2×10^5 cells/well in 6-well plates. The next day the cells were incubated with 1 μM [^{3}H]bis-POM-PMEA for 2 h in complete growth medium. The medium was removed, and the cells were put on ice and washed three times with 2 ml of ice-cold phosphate-buffered saline. Trypsin solution (0.2 ml) was applied to the plates, and the cell suspension was added to 4 ml of scintillation fluid. For efflux experiments, cells were plated in triplicate in 12-well plates at a density of 1×10^5 cells/well. The next day drug accumulation was accomplished by growth for 2 h in ATP-depletion medium consisting of glucose-free, pyruvate-free Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum, 10 mM deoxyglucose, 10 mM sodium azide, and 1 μM [^{3}H]bis-POM-PMEA. After accumulation, the cells were washed quickly with phosphate-buffered saline, and incubated with prewarmed complete medium lacking drug. Aliquots of medium were obtained over a 2-h efflux period.

Preparation of Membrane Vesicles and Transport Experiments—Membrane vesicles were prepared by the nitrogen cavitation method (31), and transport experiments were performed using the rapid filtration method essentially as described previously (32). Transport experiments were carried out in medium containing membrane vesicles (10 μg), 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 4 mM ATP, 10 mM phosphocreatine, 100 μg/ml creatine phosphokinase, and radiolabeled compounds ± unlabeled compounds, in a total volume of 50 μl. Reactions were carried out at 37 °C and stopped by the addition of 3 ml of ice-cold stop solution (0.25 M sucrose, 100 mM NaCl, 10 mM Tris-HCl, pH 7.4) Samples were passed through 0.22-μm Durapore membrane filters (Millipore, Bedford, MA) under vacuum. The filters were washed three times with 3 ml of ice-cold stop solution and dried at room temperature for 30 min. Rates of net ATP-dependent transport were determined by subtracting the values obtained in the presence of 4 mM AMP from those obtained in the presence of 4 mM ATP.

FIG. 1. **Immunoblot detection of MRP8 in transfected LLC-PK1 cells.** Cell lysates were prepared from LLC-PK1 cells transfected with parental plasmid (*lane 1*) or MRP8 expression vector (*lanes 2–4*). Proteins (50 μg/lane) were resolved by SDS-polyacrylamide gel electrophoresis on 8% gels, electrotransferred to nitrocellulose membranes, and incubated with MRP8 polyclonal antibody. The sizes of molecular weight standards (in kilodaltons) and the location of MRP8 are indicated.

TABLE I

Intracellular cAMP levels in MRP8-transfected LLC-PK1 cells

Intracellular cAMP levels in LLC-PK1 cells transfected with parental vector or with MRP8 expression vector were measured in the absence of stimulation or after 30 min of stimulation with 50 μM forskolin as described under "Experimental Procedures." The values shown represent means ± S.E. of six independent experiments, each performed in triplicate.

Treatment	LLC-PK1-pcDNA	LLC-PK1-MRP8-1	LLC-PK1-MRP8-2
	pmol/10^6 cells	*pmol/10^6 cells*	*pmol/10^6 cells*
None	6.6 ± 3.1	4.7 ± 2.1[a]	4.3 ± 2.7[a]
Forskolin	210 ± 110	138 ± 93[a]	173 ± 98[a]

[a] Values are significantly different ($p < 0.05$) from the corresponding control transfectant values as determined by the two-tailed nonparametric Wilcoxon test.

RESULTS

Ectopic Expression of MRP8 in LLC-PK1 Cells—To characterize the functional properties of MRP8, a cellular model was generated by transfecting LLC-PK1 cells with MRP8 expression vector. Immunoblot analysis indicated that MRP8 protein was expressed in several of the resulting G418-selected clones, as indicated by the intensely immunoreactive bands present in three clones transfected with MRP8 vector, but not in the parental vector-transfected control cells (Fig. 1). The apparent molecular weight of MRP8 (~170,000–190,000) was higher than its calculated molecular mass (154 kDa) and the apparent molecular weight of the protein expressed in insect cells (~155,000, data not shown), as would be expected for a glycosylated transmembrane protein. Two of these clones were selected for functional studies on MRP8.

Cellular Efflux of Cyclic Nucleotides by MRP8—To determine whether MRP8 is capable of extruding cyclic nucleotides from cells, intracellular cAMP levels were analyzed before and after stimulation with forskolin. Basal cAMP levels in the MRP8-transfected cells were consistently lower than those of the parental vector control cells (Table I). The intracellular levels in LLC-PK1-MRP8-1 and LLC-PK1-MRP8-2 cells were 4.7 ± 2.1 pmol/10^6 cells and 4.3 ± 2.7 pmol/10^6 cells, respectively, in comparison with the control cells in which the level was 6.6 ± 3.1 pmol/10^6 cells. These values corresponded to

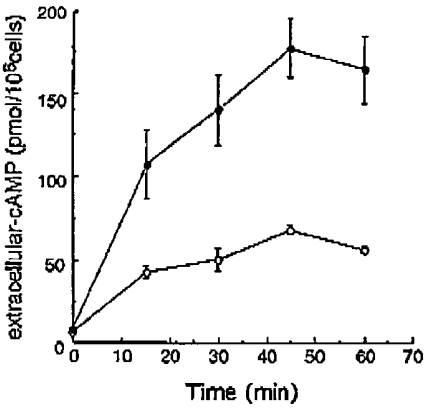

FIG. 2. **Time course of cAMP efflux following forskolin stimulation.** Parental vector-transfected (*open symbols*) and MRP8-transfected LLC-PK1 cells (*closed symbols*, LLC-PK1-MRP8-1) were stimulated with 50 μM forskolin, and the appearance of cAMP in the medium was measured as described under "Experimental Procedures." Values are means ± S.E. of a representative experiment performed in triplicate.

reductions of 29 and 35% for LLC-PK1-MRP8-1 and LLC PK1-MRP8-2, respectively. Reduced cAMP levels were also observed for the MRP8-transfected cells after stimulation with forskolin. After 30 min of stimulation with 50 μM forskolin, the cAMP levels in LLC-PK1-MRP8-1 and LLC-PK1-MRP8-2 were 34 and 18% lower than the levels in control cells (Table I).

Separate experiments were performed to analyze the appearance of cAMP in the medium following forskolin stimulation. Cellular extrusion was significantly greater for the MRP8-transfected cells at each of the time points examined (Fig. 2). At the 15-min time point, the cAMP levels in the medium of control and LLC-PK1-MRP8-1 cells were 42.9 and 108 pmol/10^6 cells, respectively. A comparable increment attributable to MRP8 was present at the 45-min time point, at which time the extracellular cAMP level was 178 pmol/10^6 cells for LLC-PK1-MRP8-1 in comparison with 68.3 pmol/10^6 for the control cells. Hence, at the 15- and 45-min time points the cAMP levels were 2.5- and 2.6-fold higher in the medium of the MRP8-transfected cells.

To assess the capabilities of MRP8 with regard to cGMP, intracellular and extracellular levels were measured before and after stimulation with SIN-1A. As shown in Table II, the intracellular cGMP levels were 2.0 ± 0.8 and 1.3 ± 0.6 pmol/10^6 cells, and the extracellular levels were 4.8 ± 1.8 and 5.0 ± 1.2 pmol/10^6 cells, respectively, for control cells and LLC-PK1-MRP8-1 cells. Thus the intracellular and extracellular cGMP levels in MRP8-transfected cells were, respectively, 35% lower and 16% higher than control cells. Following 30 min of stimulation with SIN-1A; the intracellular cGMP levels were 9.0 ± 4.5 and 5.1 ± 1.8 pmol/10^6 cell, and the extracellular levels were 26.1 ± 3.2 and 32.4 ± 5.1 pmol/10^6 cell, respectively, for the control cells and LLC-PK1-MRP8-1 cells (Table II). Hence, following stimulation, the intracellular cGMP level in LLC-PK1-MRP8-1 cells was 43% lower than the control cells, and the extracellular levels were 24% higher than the control cells. In combination, these experiments showed that expression of MRP8 results in consistent, but modest, depression in intracellular cAMP and cGMP levels, and enhancement of cyclic nucleotide extrusion.

Analysis of the Drug Sensitivity of MRP8-transfected LLC-PK1 Cells—MRP8-transfected cells exhibited increased resistance toward clinically relevant purine and pyrimidine nucleotide analogs (Table III). LLC-PK1-MRP8-1 cells were 5.4-fold resistant to the purine nucleotide analog PMEA but did not exhibit increased resistance toward three other purine nucleotide analogs, 6-thioguanine, 2′-chloro-2′-deoxyadenosine, and deoxycorfomycin. In addition, LLC-PK1-MRP8-1 cells were 6.1-fold resistant to the antiviral pyrimidine analog ddC and 2.9-fold resistant to the anticancer pyrimidine analog 5-FUra. Increased resistance was also observed for two other fluoropyrimidines of clinical significance, 5-FdUrd (5.2-fold), a ribosylated intracellular metabolite of 5-FUra, which like 5-FUra is employed as an intravenous agent, and 5-dFUrd (3.4-fold), a metabolite of the oral fluoropyrimidine capecitabine, which is further metabolized in the cell to 5-FUra. Small decreases in the sensitivity of LLC-PK1-MRP8-1 cells were observed for the pyrimidine nucleotide analogs 2′,3′-dideoxy-3′-thial-cytidine and 3′-azido-3′-deoxythymidine, but these differences did not reach statistical significance. Representative growth curves for PMEA, ddC, and 5-FUra are shown in Fig. 3. Increased resistance was not detected for several natural product anticancer agents, including vincristine, paclitaxel, doxorubicin, and etoposide (Table III). A similar drug resistance phenotype was observed for LLC-PK1-MRP8-2, which exhibited 5.6-, 7.1-, 4.1-, 3.2-, and 2.5-fold resistance toward PMEA, ddC, 5-FUra, 5-FudR, and 5-dFUrd, respectively (p values < 0.05; data not shown).

TABLE II

Intracellular and extracellular cGMP levels of LLC-PK1-MRP8-1 cells

Intracellular and extracellular cGMP levels in LLC-PK1 cells transfected with parental vector or MRP8 expression vector were measured in the absence of stimulation or after 30 min of stimulation with 50 μM SIN-1A as described under "Experimental Procedures." The values shown represent means ± S.E. of six independent experiments, each performed in triplicate.

Treatment	LLC-PK1-pcDNA		LLC-PK1 MRP8-1	
	Intracellular	Extracellular	Intracellular	Extracellular
	pmol/10^6 cells		*pmol/10^6 cells*	
None	2.0 ± 0.8	4.3 ± 1.8	1.3 ± 0.6[a]	5.0 ± 1.2[a]
SIN-1A	9.0 ± 4.5	26.1 ± 3.2	5.1 ± 1.8[a]	32.4 ± 5.1[a]

[a] Values are significantly different ($p < 0.05$) from the corresponding control transfectant values as determined by the two-tailed nonparametric Wilcoxon test.

TABLE III

Drug sensitivity analysis of MRP8-transfected LLC-PK1 cells

The drug sensitivities of LLC-PK1 cells transfected with parental vector or MRP8 expression vector were measured in 3-day growth assays as described under "Experimental Procedures." The IC_{50} values are the drug concentrations that inhibited growth by 50% and represent means ± S.E. of at least four independent experiments, each performed in triplicate. Fold resistance is enumerated at the IC_{50} of LLC-PK1-MRP8-1 divided by the IC_{50} of the parental vector transfected control cell line. 6-TG, 6-thioguanine; CdA, 2′-chloro-2′-deoxyadenosine; DCF, deoxycorfomycin; 3TC, 2′,3′-dideoxy-3′-thial-cytidine; AZT, 3′-azido-3′-deoxythymidine; VCR, vincristine; DOX, doxorubicin; ETOP, etoposide.

Agent	IC_{50}		-Fold resistance
	LLC-PK1-pcDNA	LLC-PK1-MRP8-1	
	μM		
PMEA	457 ± 79	2463 ± 336	5.4[a]
6-TG	500 ± 41	546 ± 100	1.1
CdA	8.1 ± 2.7	8.2 ± 4.6	1.0
DCF	256 ± 52	263 ± 50	1.0
3TC	869 ± 56	1078 ± 48	1.4
AZT	507 ± 86	639 ± 41	1.3
ddC	384 ± 52	2430 ± 155	6.1[a]
5-FUra	8.8 ± 3.0	26.2 ± 12	2.9[a]
5-FdUrd	20.3 ± 9.0	105 ± 39	5.2[a]
5-dFUrd	29.0 ± 8.6	98.7 ± 35	3.4[a]
VCR	0.09 ± 0.01	0.10 ± 0.02	1.1
Taxol	0.35 ± 0.07	0.37 ± 0.05	1.1
DOX	0.42 ± 0.03	0.45 ± 0.1	1.1
ETOP	0.92 ± 0.04	1.21 ± 0.10	1.2

[a] Values are significantly different from the control transfectant ($p < 0.05$) as determined by the nonparametric two-tailed Wilcoxon test.

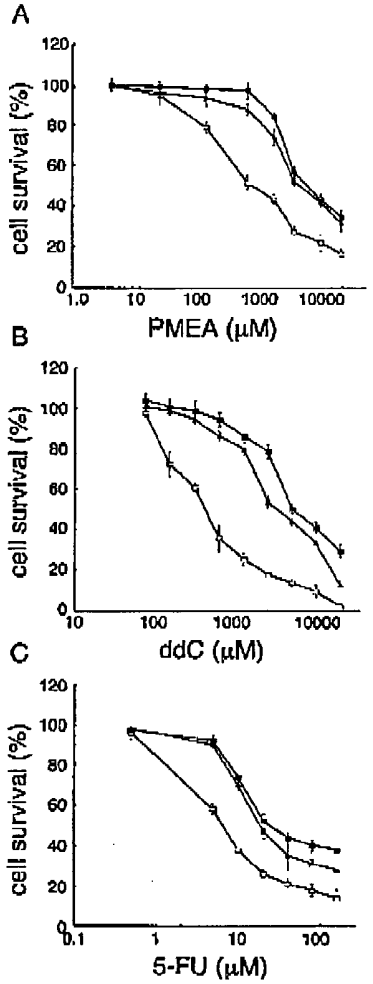

FIG. 3. **Sensitivity of MRP8-transfected and parental vector-transfected LLC-PK1 cells to PMEA, ddC, and 5-FUra.** The sensitivity of parental vector-transfected (*open squares*) and MRP8-transfected LLC-PK1 cells (*upright triangles*, LLC-PK1-MRP8-1; *closed squares*, LLC-PK1-MRP8-2) to PMEA (*A*), ddC (*B*), and 5-FUra (*C*) was analyzed using the tetrazolium salt microtiter plate assay as described under "Experimental Procedures." Values are means ± S.E. of representative experiments performed in triplicate.

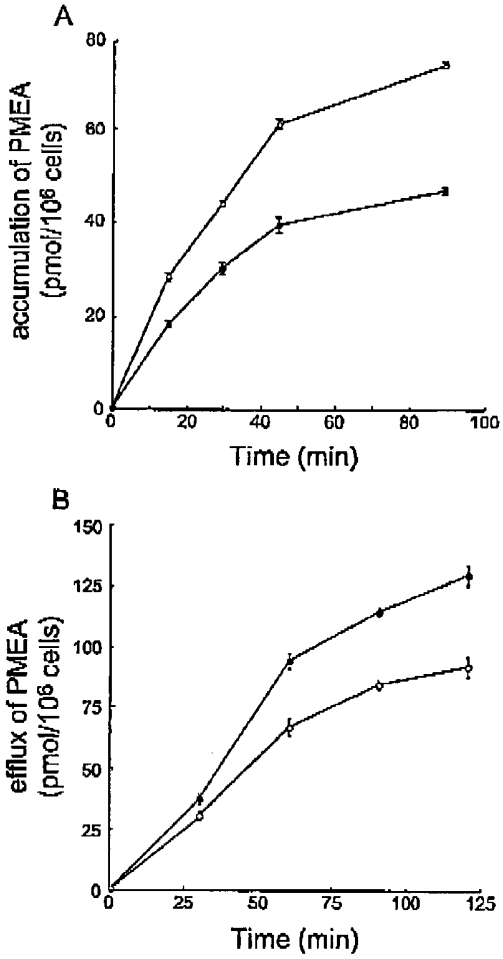

FIG. 4. **Cellular accumulation and efflux of [³H]bis-POM-PMEA.** *A*, time course of accumulation of [³H]bis-POM-PMEA in parental vector-transfected (*open symbols*) and MRP8-transfected (*closed symbols*, LLC-PK1-MRP8-1) LLC-PK1 cells. Cells were incubated in 1 μM [³H]bis-POM-PMEA, and intracellular radioactivity was measured at various time points. *B*, time course of the efflux of radioactivity into medium. Parental vector-transfected LLC-PK1 cells and LLC-PK1-MRP8-1 cells were incubated in the presence of 1 μM [³H]bis-POM-PMEA for 2 h under energy-depletion conditions as described under "Experimental Procedures," and the medium was changed to complete medium lacking drug. Efflux of radioactivity into the medium was then measured at various time points. Values are means ± S.E. of representative experiments performed in triplicate.

Analysis of PMEA Accumulation and Efflux—The effect of MRP8 on the cellular kinetics of a representative agent was analyzed. To this end we employed bis-POM-PMEA, an uncharged bis-ester prodrug of PMEA that is more effective than the parent compound in crossing the plasma membrane (33). Once inside the cell the pivaloyloxymethyl moiety of bis-POM-PMEA is cleaved to release free PMEA. LLC-PK1-MRP8-1 exhibited reduced accumulation of [³H]bis-POM-PMEA compared with parental vector-transfected cells (Fig. 4*A*). After 15 min of incubation in growth medium containing 1 μM [³H]bis-POM-PMEA, drug accumulation in LLC-PK1-MRP8-1 cells was ~60% of the control cells. This difference in accumulation was maintained throughout the time course of the assay. Separate efflux experiments were performed under conditions in which initial intracellular drug levels were comparable in the two cell lines, by first allowing accumulation of 1 μM [³H]bis-POM-PMEA to proceed under energy-depletion conditions. Following a 2-h incubation period, the growth medium was replaced with complete medium lacking drug, and efflux of radiolabeled drug into the medium was measured. As shown in Fig. 4*B*, LLC-PK1-MRP8-1 exhibited enhanced drug efflux by comparison with the control cells. At the 2-h time point, efflux by the MRP8-transfected cells was 40% greater than the control cells.

Transport of 5′-Fluoro-2′-deoxyuridine Monophosphate—By contrast with cyclic nucleotides and PMEA, both of which are amphipathic anions, 5-FUra is an uncharged pyrimidine analog. The possibility that MRP8 confers resistance to this agent, as well as to 5-FdUrd and 5-dFUrd, by transporting 5-FdUMP, the anionic cytotoxic metabolite of these compounds as opposed to the parent compounds, was therefore considered. This was examined by analyzing the ability of the pump to transport 5-FUra, 5-FudR, and 5-FdUMP into inside-out membrane vesicles. As shown in Fig. 5, although membrane vesicles prepared from parental vector transfected cells were able to catalyze the MgATP-dependent transport of [³H]5-FdUMP, an increment attributable to MRP8 was consistently observed. MgATP-de-

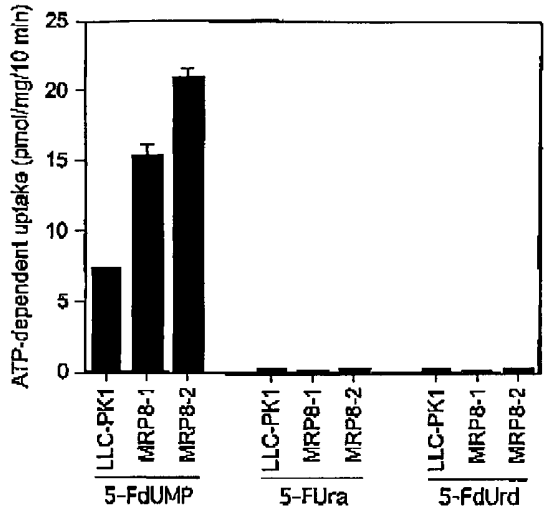

FIG. 5. **MgATP-dependent uptake of [^{3}H]5′-fluorouracil, [^{3}H]5′-fluorodeoxyuridine, and [^{3}H]5′-fluorodeoxyuridine monophosphate into inside-out membrane vesicles.** Membrane vesicles (10 μg) prepared from parental vector-transfected LLC-PK1 cells or LLC-PK1-MRP8-1 and LLC-PK1-MRP8-2 cells were incubated for 10 min at 37 °C in uptake medium containing 4 mM ATP or 4 mM AMP and the indicated radiolabeled compounds. MgATP-dependent uptake for 1 μM [^{3}H]5-FdUMP, 1 μM [^{3}H]5-FUra, or 1 μM [^{3}H]5-FdUrd was calculated by subtracting the values obtained in transport medium containing MgAMP from the values obtained in medium containing MgATP. Values shown are means ± S.E. of a representative experiment. This experiment was repeated at least five times with similar results.

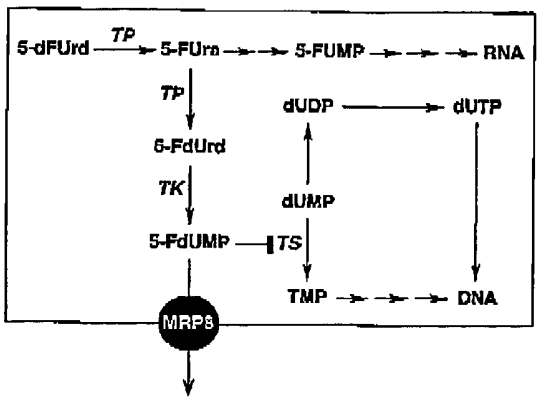

FIG. 6. **Schematic diagram depicting the role played by MRP8 in conferring cellular resistance to fluoropyrimidines.** 5-FUra, 5-FdUrd, and 5-dFUrd are converted to the cytotoxic intracellular metabolite 5-FdUMP. 5-FdUMP, in the presence of the reduced folate cofactor 5,10-methylene tetrahydrofolate, forms an inhibitory complex with thymidylate synthase (*TS*), which blocks the *de novo* synthesis of thymidine monophosphate. This block leads to depletion of thymidine pools required for DNA synthesis, and misincorporation of dUTP into DNA consequent to elevated levels of this nucleotide. MRP8 is able to mediate the transport of 5-FdUMP. *TP*, thymidine phosphorylase; *TK*, thymidine kinase.

pendent uptake for LLC-PK1-MRP8-1 and LLC-PK1-MRP8-2 was 15.4 and 21.0 pmol/mg/10 min, respectively, whereas uptake for the control membranes was only 7.3 pmol/mg/10 min (Fig. 5). By contrast, with [^{3}H]5-FdUMP, MgATP-dependent uptake of [^{3}H]5-FUra and [^{3}H]5-FdUrd was negligible for both MRP8-enriched and control membrane vesicles.

DISCUSSION

In the present study the functional properties of MRP8 were analyzed by the use of transfected LLC-PK1 cells. In combination, the results showing that MRP8 is able to depress intracellular levels of cAMP and cGMP by enhancing cellular extrusion, confer resistance to PMEA, and transport 5-FdUMP provide the first evidence that this protein functions as a lipophilic anion pump. These results indicate that the cellular extrusion of cyclic nucleotides, a phenomenon that has been well documented in numerous types of mammalian cells, is accomplished by a plasma membrane system that is composed of at least three pumps. Although its substrates have yet be determined, the high degree of structural resemblance between MRP9 and MRP8 (27, 28) suggests that it may also participate in this process.

Although cellular extrusion of cyclic nucleotides from mammalian cells is a well established phenomenon, it has not been considered a major factor in attenuating the elevation of these second messengers consequent to the activation of cyclases. This process is thought to be mediated primarily by the action of phosphodiesterases, a view based upon the notion that an extremely rapid, high capacity system is required for the precise time-sensitive signaling mediated by cyclic nucleotides. By comparison with the enzymatic breakdown of cyclic nucleotides by phosphodiesterases, cellular efflux is thought to be low capacity, and more importantly, relatively slow. The identification of efflux pumps capable of mediating this process (this study and Refs. 18 and 19) has provided the molecular tools to investigate directly their involvement in these and related processes. The results, showing that intracellular levels of cAMP and cGMP under basal or stimulated conditions were only modestly depressed (1.2–1.8-fold) by ectopic expression of MRP8, tend to support the view that efflux pumps are not potent attenuation factors, as do previous reports showing that ectopic expression of MRP4 and MRP5 is similarly associated with modest effects on intracellular cyclic nucleotide levels (34, 35). These studies, in combination with measurements showing that the latter two pumps have reasonably high affinities for cGMP, and in the case of MRP4, for cAMP (18, 19), suggest that it is the high efficiency of the phosphodiesterase system that limits the impact of efflux pumps as opposed to the inability of pumps to function at physiological concentrations of these second messengers. Further studies should help to determine the circumstances and extent to which efflux pumps participate in intracellular cyclic nucleotide homeostasis and also to define their involvement in the physiological processes in which extruded cyclic nucleotides have been proposed as primary messengers.

Analysis of the drug sensitivity of MRP8-transfected LLC-PK1 cells showed that MRP8 is able to confer resistance to fluoropyrimidines, ddC, and PMEA. However, resistance toward 6-thioguanine, an agent that is part of the resistance profiles of MRP4 and MRP5, was not detected (19, 25). Fluoropyrimidines, which are a mainstay in the treatment of colon cancer and are also active in breast and head and neck cancer, are among the most widely employed anticancer agents. The cytotoxicity of 5-FUra is mediated predominately by its intracellular metabolite, 5-FdUMP, which in combination with 5,10-methylene tetrahydrofolate forms a stable inhibitory complex with thymidylate synthase. The biochemical consequences of this block, depletion of thymidine nucleotides and build-up of dUTP, engender impaired DNA synthesis and misincorporation of uracil into DNA, respectively. Cellular resistance factors for fluoropyrimidines include increased expression of thymidylate synthase, decreased expression of enzymes involved in metabolic activation, and increased expression of dUTPase (36–41). Our results indicate that MRP8 is a potential clinical resistance factor for fluoropyrimidines and that it confers resistance to this class of agents by mediating the efflux of 5-FdUMP, the intracellular cytotoxic metabolite of 5-FUra,

5-FudR, and the oral fluoropyrimidine capecitabine (Fig. 6). The presence of significant levels of 5-FdUMP transport by membranes prepared from control LLC-PK1 cells (Fig. 6), in which MRP8 is barely detectable (Fig. 1), suggests that pumps other than MRP8 may also be involved in this process. Although, to the best of our knowledge, increased efflux of fluoropyrimidine metabolites has not been described in drug-resistant cell lines, this potential mechanism warrants more detailed analysis in view of our findings. The ability of MRP8 to confer resistance to the anti-AIDS nucleotide analog ddC is also noteworthy. We infer that this activity is consequent to the efflux of the intracellular nucleotide metabolites of this agent, by analogy with the mechanisms by which MRP8 confers resistance to fluoropyrimidines, and MRP4 and MRP5 confer resistance to and 6-mercaptopurine (42). Investigations of clinical resistance to ddC have focused primarily on mutations in the human immunodeficiency virus reverse transcriptase and alterations in the levels of cellular metabolizing enzymes (43). Our results suggest that cellular efflux is a potential clinical resistance for this agent. A recent study showing that a drug-resistant cell line in which ABCG2 (breast cancer resistance protein (BCRP)) is over-expressed is cross-resistant to 3′-azido-3′-deoxythymidine suggests that ABC transporters that are not MRP family members may also be involved in resistance to this class of agents (44), although this remains to be confirmed in experiments using recombinant ABCG2. Analysis of the expression of MRP8 in clinical samples should help to determine its importance in clinical resistance to fluoropyrimidines, ddC, and the anti-hepatitis B agent PMEA.

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